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The parasporal crystals of *Bacillus pumilus* strain 15.1: a potential virulence factor?

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Running Title: Parasporal crystal in *Bacillus pumilus* 15.1

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25 **Abstract**

26 *Bacillus pumilus* strain 15.1 was previously found to cause larval mortality in the Med-fly
27 *Ceratitis capitata* and was shown to produce crystals in association with the spore. As
28 parasporal crystals are well-known as invertebrate-active toxins in entomopathogenic
29 bacteria such as *Bacillus thuringiensis* (Cry and Cyt toxins) and *Lysinibacillus sphaericus* (Bin
30 and Cry toxins), the *B. pumilus* crystals were characterised. The crystals were composed of a
31 45 kDa protein that was identified as an oxalate decarboxylase by peptide mass
32 fingerprinting, N-terminal sequencing and by comparison with the genome sequence of strain
33 15.1. Synthesis of crystals by a plasmid-cured derivative of strain 15.1 (produced using a
34 novel curing strategy), demonstrated that the oxalate decarboxylase was encoded
35 chromosomally. Crystals spontaneously solubilized when kept at low temperatures and the
36 protein produced was resistant to trypsin treatment. The insoluble crystals produced by
37 *B. pumilus* 15.1 did not show significant toxicity when bioassayed against *C. capitata* larvae,
38 but once the OxdD protein was solubilized, an increase of toxicity was observed. We also
39 demonstrate that the OxdD present in the crystals has oxalate decarboxylase activity as the
40 formation of formate was detected, which suggests a possible mechanism for *B. pumilus* 15.1
41 activity. To our knowledge, the characterization of the *B. pumilus* crystals as oxalate
42 decarboxylase is the first report of the natural production of parasporal inclusions of an
43 enzyme.

44

45 **Introduction**

46 The production of spore-associated (parasporal) crystals by several species of bacteria within
47 the genus *Bacillus* and related genera is well known. These proteins are almost always
48 entomopathogenic toxins, active against a wide range of invertebrate targets (Bechtel and
49 Bulla, 1976; 2007) although crystals without known targets (sometimes termed parasporins)
50 are also known. Such parasporins are related in sequence and structure to known

51 invertebrate-active toxins and it is likely that their natural target merely remains to be
52 discovered (although activity against certain human cancer cells in culture has been reported
53 (Ohba et al., 2009)). The most studied proteinaceous toxins are the Cry and Cyt toxins,
54 produced mainly by *Bacillus thuringiensis* (*Bt*), which are the principal agents responsible for
55 the toxicity of these bacteria toward insects. The insecticidal activity of crystal proteins
56 produced by *Bt* has been extensively used as the basis of many commercial products. The
57 ability to produce parasporal crystals is not restricted to *Bt* as some strains of *Lysinibacillus*
58 *sphaericus* (Jones et al., 2007), *Clostridium bifermentans* (Barloy et al., 1996), *Paenibacillus*
59 *popilliae* (Zhang et al., 1997), *Brevibacillus laterosporus* (Smirnova et al., 1996) and *P.*
60 *lentimorbus* (Yokoyama et al., 2004), also produce parasporal inclusions active against insects.
61 The mechanisms of action proposed for the parasporal crystal toxins generally require
62 solubilization and proteolytic activation of the protoxin form in the midgut of the target
63 invertebrate (Haider et al., 1986; Palma et al., 2014). Serine proteases are important in both
64 solubilization and activation of *Bt* protoxins and, in some insects, changes in the protease
65 profile of their guts have been associated with resistance to *Bt* toxin (Li et al., 2004;
66 Karumbaiah et al., 2007).

67 Our research group reported a *Bacillus pumilus* strain toxic toward the Mediterranean fruit
68 fly, *Ceratitis capitata* (Molina et al., 2010). Previous assays showed that the toxicity of
69 *B. pumilus* 15.1 can be inactivated either by heat or by proteases, suggesting that the virulence
70 factor produced by this strain could be proteinaceous (Molina, 2010). Since its initial isolation
71 and testing, our strain appears to have decreased in toxicity, even though it continues to
72 produce the parasporal crystals, mainly composed of a 45 kDa protein, that we have
73 previously described (Garcia-Ramon et al., 2016). Despite the loss of toxicity, the crystal
74 protein was still considered as a candidate toxin (possibly interacting with another factor,
75 now lost or under-expressed). In this work we describe the characterization of the crystal

76 inclusions produced by *B. pumilus* 15.1 as the first example of a parasporal enzyme crystal
77 and we propose a potential mechanism of action for this entomopathogenic strain.

78

79

80 **Results**

81 ***Identification of the crystal protein***

82 The spore-crystal complex of a *B. pumilus* 15.1 culture, sporulated in T3 medium was used to
83 isolate crystals using sucrose density gradient centrifugation (Garcia-Ramon et al., 2016).
84 Crystals formed bands at the interface formed between the solutions of 72% and 79% sucrose
85 (like many Cry toxins (Thomas and Ellar, 1983; Koller et al., 1992; Jones et al., 2007; Swiecicka
86 et al., 2008)). They were also found at the 79% / 84% sucrose interface. The enriched crystal
87 proteins from both bands produced a major band of 45 kDa on SDS-PAGE, as previously
88 observed (Garcia-Ramon et al., 2016) and this was excised for fingerprint analysis using
89 MALDI-TOF MS. Mass spectrometry of the intact protein revealed a mass of 43,799 Da.
90 Comparison of mass peaks obtained from fingerprinting with the recently published
91 *B. pumilus* 15.1 genome (Garcia-Ramon et al., 2015a) and *Bacillus* databases produced
92 matches (40.8% sequence coverage) with OxdD, a putative oxalate decarboxylase encoded in
93 Contig 4 of the *B. pumilus* 15.1 strain genome and an OxdD from *B. pumilus* ATCC 7061. The
94 predicted MW of this *B. pumilus* 15.1 OxdD protein was 43,799.1 Da, corresponding to the
95 molecular weight determined by MS. The N-terminal analysis of this ~45 kDa protein, after
96 treatment with trypsin (see below), rendered the sequence S-E-K-P-D/N-G-I-P. The
97 SEKPNGIP sequence showed 100% identity and 100% sequence coverage with oxalate
98 decarboxylase from *B. pumilus* 15.1 (accession number KLL01117) and other *B. pumilus*
99 strains (KIL13977) from their second amino acid (the initiator methionine was missing as
100 frequently occurs with *in vivo* methionine aminopeptidase activity, particularly when the next
101 residue is small, such as the Ser residue in this case (Xiao et al., 2010).

102 The 45 kDa protein was also subjected to 2D electrophoresis for protein characterization
103 (Figure 1) and two spots at approximately pI 5.5 (spot A) and pI 10 (spot B) were observed.
104 Both spots were analysed by MALDI-TOF MS and identified as oxalate decarboxylase. The
105 theoretical pI of oxalate decarboxylase is 5.22, which corresponds with the pI observed for
106 spot A. The appearance of spot B at pI 10 is unexplained since this does not fit with the
107 theoretical value and, as far as we know, there is no reported oxalate decarboxylase with a pI
108 ≥ 10 in the literature.

109 Taken together, the data above indicate that the 45 kDa protein is that encoded by the
110 *B. pumilus* strain 15.1 *oxdD* gene and the protein will be described from this point as oxalate
111 decarboxylase. Features of this protein family, including the two Mn^{2+} binding sites, are
112 conserved in the *B. pumilus* protein and we were able to construct a molecular model of the
113 protein based on the known structure of the *B. subtilis* enzyme (PDB accession 5HI0) using the
114 Swiss model program (Schwede et al., 2003) as shown in Figure 2.

115

116 ***The oxdD gene***

117 The *oxdD* gene is located on Contig 4 of the draft *B. pumilus* 15.1 genome. Analysis of the
118 region upstream of this gene using the DBTBS database of transcription factors in *B. subtilis*
119 (Sierro et al., 2008) predicts that the gene is preceded by a putative sigma K promoter
120 (GGCCTTTTGTACCTCACACCATACGATG) beginning 47 nt upstream of the initiator ATG.
121 Regulation by this late mother cell sigma factor would be consistent with previous studies that
122 demonstrated that *B. pumilus* strain 15.1 produces the crystal protein during sporulation when
123 cultured in T3 medium, showing a maximal accumulation after 72 h (Garcia-Ramon et al., 2016).
124 Sigma K is also used in the production of some Cry proteins in *Bt* (reviewed in (Deng et al.,
125 2014)). In addition, beginning 80 nt upstream of the ATG is a putative MntR transcription
126 factor site (GTTTCACCTTATGAAAACG). This site is normally associated with regulation of
127 Mn^{2+} transport with repression of *mntH* at high Mn^{2+} concentrations. The Mn^{2+} ion is the only

128 trace element present in T3 medium with a standard concentration of 5 mg/L of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$
129 (25 μM), so we analyzed the accumulation of oxalate decarboxylase at concentrations ranging
130 from 0 to 0.5 g/L (0 to 2.5 mM). The cultures all reached comparable cell densities at the end of
131 the incubation period and the results showed (Figure 3) that oxalate decarboxylase was present
132 at all Mn^{2+} concentrations tested, showing maximal accumulation at 0.5 mg/L and 5 mg/L of
133 MnCl_2 (Figure 3, lanes 1 and 2). The variation of oxalate decarboxylase seen in these
134 experiments may be due to variations in expression, possibly mediated via the putative MntR
135 region. Alternatively, the stability of the oxalate decarboxylase could also be involved since the
136 Mn^{2+} binding sites are conserved in the *B. pumilus* 15.1 protein (Figure 2). However, we might
137 expect stabilisation to be greater at higher Mn^{2+} concentrations, which is the opposite to the
138 effect seen in our experiments.

139

140 ***The oxalate decarboxylase protein shows unexpected solubilisation behaviour***

141 When protein crystals are formed, subsequent solubilisation can be expected to occur to
142 release their potential (as occurs with crystal toxins). The crystal toxins of Bt often solubilise
143 at pH values ≥ 9.0 , so solubility of the oxalate decarboxylase crystals was tested under similar
144 conditions. In our standard procedure, crystals from the sucrose gradient, washed with PBS,
145 were resuspended in milliQ water and kept at -20°C . When crystals were used, an aliquot of
146 the thawed crystal suspension was centrifuged, the supernatant was discarded and the pellet
147 resuspended for 1 h at 37°C in 0.1 M sodium phosphate pH 9.0. After this time, samples were
148 centrifuged and soluble and insoluble fractions were analysed by SDS PAGE. Approximately
149 50% of the crystal protein was solubilized at pH 9.0 (results not shown) but total protein
150 content (soluble and insoluble) was considerably lower than expected. Reanalysis of the
151 stored sample revealed that the protein content of the crystals kept at -20°C (pellet fraction)
152 decreased over time, with the crystals of oxalate decarboxylase protein becoming solubilized
153 into the supernatant fraction on low temperature storage. To verify this phenomenon, a fresh

crystal preparation was divided into two fractions. One was kept at -20°C and the second at room temperature (RT). Ten microliter samples were taken over time from each aliquot, centrifuged, pellet and supernatant separated, and analyzed by SDS-PAGE gels. The results presented in Figure 4 showed that when the crystal preparation was kept at RT the oxalate decarboxylase was observed only in the pellet fractions (Figure 4A). In contrast, when the sample was kept at -20°C, the concentration of oxalate decarboxylase in the supernatant fraction increased as the incubation time at -20°C progressed (Figure 4B). Transmission electron microscopic analysis of crystals revealed that the sample incubated at RT contained parasporal crystals, while the sample incubated at -20°C (for longer than 24 h) showed almost no crystals at all (data not shown). The protein from the pellet and supernatant fractions obtained after incubation at -20°C were identified by MALDI-TOF MS and LC-MS/MS (soluble fraction only) to rule out the possibility that other proteins may have been present in the crystals. Once again, MALDI-TOF results identified only oxalate decarboxylase, in the pellet (36% coverage) and in the supernatant (34% coverage). Size exclusion chromatography of the soluble protein indicates that the protein exists in solution in a multimeric form with the protein eluting from the column at a volume, compared to molecular weight standards, consistent with a hexameric assembly (Figure 1S supporting information).

171

The oxalate decarboxylase protein is resistant to trypsin.

The soluble protein (obtained by incubation of the crystals at -20°C) was digested with a range of proteases to determine whether the protein was susceptible to their action or was (partially) resistant (as would be expected, eg for Cry toxins). Trypsin, chymotrypsin, papain and “Proteinase from *B. subtilis*” were tested at a 10:1 ratio (w/w) protein:enzyme. SDS-PAGE analysis revealed that the oxalate decarboxylase protein was completely digested by papain and “Proteinase from *B. subtilis*” (Figure 5, Panel A, lanes 4 and 5), while trypsin and chymotrypsin gave no visible digestion at this protein:enzyme ratio (Figure 5, Panel A, lanes 2

180 and 3 respectively). Increasing the quantity of chymotrypsin (1:1 and 1:10 protein:enzyme),
181 produced increasing degradation of the oxalate decarboxylase (Figure 5, Panel C) but
182 protein:trypsin ratios of 1:1 to 1:500 still produced no change in the band (Figure 5, Panel B,
183 lanes 2-6) while this enzyme was able to activate solubilized Cry1Aa13 used as control to
184 produce the expected 66 kDa product (Figure 5, Panel D).

185

186 ***Investigating the location of the *oxdD* gene in *B. pumilus* 15.1***

187 The majority of crystal toxin genes of *Bt* are encoded on extrachromosomal elements and we
188 decided to investigate the location of the *oxdD* gene. We have recently shown that the
189 *B. pumilus* 15.1 strain bears one plasmid of 7,785 bp named pBp15.1S (Contig 38) and one
190 megaplasmid of unknown size named pBp15.1B (Garcia-Ramon et al., 2015b). The *oxdD* gene
191 was found in Contig 4 (Accession number LBDK01000004), a contig of 57,329 bp that encodes
192 51 predicted proteins. This contig is distinct from the small plasmid pBp15.1S but has a size
193 that could either represent part of a megaplasmid or a chromosome fragment. In order to
194 determine if the crystals produced by *B. pumilus* 15.1 strain were encoded by the
195 chromosome or the megaplasmid we decided to cure the strain of its extrachromosomal
196 elements.

197

198 ***Obtaining *B. pumilus* 15.1 variants without extrachromosomal elements***

199 Different methodologies described in the literature for curing extrachromosomal elements
200 such as heat and SDS treatment, acridine orange and promethazine treatment (detailed in the
201 Materials and Methods section) were used without any success (data not shown).

202 In a previous characterization of the *B. pumilus* 15.1 strain under electron microscopy (Garcia-
203 Ramon et al., 2016) we observed that the strain showed a particularly thick cell wall. We
204 hypothesized that the lack of effect of the compounds tested for plasmid curing might be
205 caused by the difficulty that these compounds might encounter in penetrating the cells to

interfere with plasmid replication. For that reason, we designed a strategy in order to improve the success of compound internalization and hence the success of plasmid curing. The strategy consisted of obtaining spheroplasts from *B. pumilus* 15.1 with the use of lysozyme prior to the treatment with the replication-interfering compounds. We tested our hypothesis with acridine orange and promethazine, two very well known curing compounds. *B. pumilus* 15.1 spheroplasts were obtained from vegetative cells as detailed in the Materials and Methods section and then they were diluted in LB medium containing acridine orange (0.03%) or promethazine (0.12%). As controls, the same amount of vegetative cells, without the lysozyme treatment, were treated under the same conditions in the presence of the replication-interfering compounds. When total DNA was extracted from one colony obtained from each treatment (Figure 6) no extrachromosomal elements were observed in those cells previously treated with lysozyme (Figure 6, lanes 3 and 4). In contrast, those cells not treated with lysozyme (Figure 6, lanes 5 and 6) showed the presence of extrachromosomal elements in their cytoplasm. The use of the spheroplasts instead of the vegetative cells seems to improve the efficiency of acridine and promethazine in curing the strain *B. pumilus* 15.1. The acridine orange strain was selected for further studies and named *B. pumilus* 15.1C (cured from plasmid (pBp15.1S) and megaplasmid (pBp15.1B)). Since, in contrast to the megaplasmid, the smaller pBp15.1S plasmid has been completely characterised and its copy number was found to be 33 (Garcia-Ramon et al., 2015b), we were able to verify its absence by PCR since, using the same methodology: no amplification was obtained from *B. pumilus* strain 15.1C (data not shown). Southern blot analysis using a Dig-labeled probe designed in the *orf7* of the plasmid pBp15.1S was also carried out. The probe hybridises to the smaller band in the gel, corresponding to the small plasmid and also interacts with the chromosomal band, most likely due to entanglement of the plasmid with chromosomal DNA. No signal (for either band) was observed in the lane corresponding to total DNA from cured *B. pumilus* 15.1C (Figure 7 Panel B, lane 2), verifying the absence of plasmid pBp15.1S.

232

233 ***The gene encoding oxalate decarboxylase in B. pumilus 15.1 has a chromosomal location***

234 The protein profile of the pellet fraction of a 72 h culture of the cured strain *B. pumilus* 15.1C
235 was obtained, analyzed by SDS-PAGE and compared to the *B. pumilus* 15.1 protein profile
236 previously described (Garcia-Ramon et al., 2016). Although the general pattern of proteins
237 was conserved, two main differences were observed: *i*) the accumulation of the 45 kDa
238 oxalate decarboxylase seems to be higher in the cured strain compared to the wild type
239 (Figure 8) and *ii*) an approximately 17 kDa protein was missing in the cured strain compared
240 to the wild-type (Figure 8, lower white arrow).

241 A MS fingerprinting analysis of the 17 kDa protein treated with trypsin produced two amino-
242 acid sequences (VLPAAGTYTFR and FYAEDTLDIQTRPVVVTPPDPCGC) both showing identity
243 with the product of the *yuaB* gene from *B. pumilus* 15.1 localized in Contig 48 and with the
244 hypothetical protein BPUM_1610 of *B. pumilus* SAFR-032 (accession number ABV62292.1).
245 The coverage of the sequence was around 19%, the predicted molecular weight of the 175 aa
246 protein was 19,297 Da including a predicted signal peptide of 27 aa, the removal of which
247 would yield a 16.3 kDa protein, consistent with the size observed in SDS PAGE gels. This
248 protein shows 67% identity with the *Bacillus subtilis* BslA protein; a protein with an
249 immunoglobulin-like fold that forms a hydrophobic coat on biofilms (Hobley et al., 2013;
250 Bromley et al., 2015).

251 Taking these results together, we can conclude that the oxalate decarboxylase of *B. pumilus*
252 15.1 is not encoded by the megaplasmid pBp15.1B, as it is expressed in the cured strain
253 *B. pumilus* 15.1C, and, therefore, the *oxdD* gene is localized in the chromosome. We can also
254 conclude that it is highly probable that the gene encoding the 17 kDa BslA-like protein is
255 present in the megaplasmid pBp15.1B as the protein does not express in the cured strain. In
256 order to prove this, two primers based on the gene *yuaB* in the strain 15.1 genome (Garcia-
257 Ramon et al., 2015a) were designed. A 727 bp product was detected only when DNA from the

258 wild type strain was used as template, but not when total DNA from *B. pumilus* 15.1C was used
259 (data not shown). As the *yuaB* gene is not present in the known sequence of pBp15.1S
260 (Garcia-Ramon et al., 2015b) and as the strain contains only one plasmid and one
261 megaplasmid, we must conclude that *yuaB* gene is present in the megaplasmid pBp15.1B. The
262 24,079 bp Contig 48 (LBDK01000048), where the *yuaB* gene is present must therefore, be
263 part of this megaplasmid and contains 27 CDSs, most of them encoding hypothetical proteins.
264 When *B. pumilus* 15.1C was analyzed under transmission electron microscopy no
265 morphological differences were observed compared to *B. pumilus* 15.1 strain (data not
266 shown). The only remarkable difference was that the number of crystals in *B. pumilus* 15.1C
267 cultures was higher than in *B. pumilus* 15.1. A quantification of the number of crystals and
268 spores from different fields of the micrographs obtained, showed that the ratio crystals:spore
269 observed in a culture of *B. pumilus* 15.1C was 0.17:1 compared to the ratio 0.09:1 previously
270 determined for *B. pumilus* 15.1 (Garcia-Ramon et al., 2016). This result seems to indicate that
271 the production of the crystals in the cured strain was higher (almost double) than in the wild
272 type strain, a fact that is in agreement with the observation from SDS-PAGE that the
273 expression of the oxalate decarboxylase protein is higher in the cured strain (Figure 8).

274

275 ***Purified and insoluble crystals produced by B. pumilus 15.1 are not toxic***

276 The crystal bands from sucrose gradients obtained from the wild type *B. pumilus* 15.1,
277 containing the majority of the oxalate decarboxylase, were tested in bioassays against first-
278 instar larvae of *C. capitata* using deionized water as negative control. As stated above, the
279 activity of strain 15.1 has decreased since initial isolation but it is possible that the purified
280 crystal, assayed at high concentrations might produce an increase in toxicity. When bioassayed
281 (Table 1) crystals obtained from *B. pumilus* 15.1 showed a mortality of only 4.2% compared to
282 that obtained in the negative control (6.25% mortality). We then tested the activity of the
283 crystal fractions after being frozen at -20°C for 4 hours to promote solubilization, performing

284 bioassays with the pellet and supernatant separately. The pellet fraction of *B. pumilus* 15.1
285 caused 6.79% mortality, while supernatant caused 18.8%. In the negative control, where just
286 water was bioassayed, a mortality of 2.08% was recorded. We observed that solubilised
287 crystals were slightly more toxic (3 fold) than the non-solubilised protein, even though a very
288 short period of time for solubilisation was allowed (only 4 h). These results may indicate that
289 oxalate decarboxylase could be involved in toxicity and it needs to be in a soluble form to
290 exert its action.

291

292 ***Oxalate decarboxylase is enzymatically active and produces formate from oxalate.***

293 With the objective of demonstrating if the oxalate decarboxylase produced by *B. pumilus* 15.1
294 as inclusion crystals is enzymatically active, two different enzymatic assays were set up. In the
295 first assay, the oxalate decarboxylase activity assay kit (Sigma Aldrich) was used to assay
296 approximately 1 µg of solubilised crystal protein. The *B. pumilus* protein produced
297 approximately 7 times more formate than the positive control enzyme (7 µl) provided with
298 the kit (9.27 and 1.25 nmol formate respectively). In the second assay, *B. pumilus* 15.1
299 crystals were purified in a sucrose gradient, resuspended in Mili Q water, kept at -20°C for 96
300 h for solubilisation and quantified by the Bradford method. Five or ten micrograms of soluble
301 protein were included in the enzymatic assays using sodium oxalate as a substrate. The
302 activity of the enzyme was evaluated in the presence and absence of Mn²⁺ (as this ion is a
303 cofactor for the enzyme). After stopping the reaction, the production of formate was analysed
304 by ¹H-NMR. For quantification purposes, 5 mM methanol was added to each sample as an
305 internal reference just before the ¹H-NMR spectra were obtained. The spectra are detailed in
306 Figure 2S as supporting information. Formate production was detected as a singlet at 8.40
307 ppm in all the spectra. After integrating the area of the formate peak and comparing with the
308 area of the methanol signal (3.31 ppm), the concentration of formate was estimated (Table 2).
309 Enzymatic assays containing 10 µg of the enzyme produced twice the amount of formate as

310 those containing 5 µg enzyme. When the enzyme was not included in the assay, formate was
311 not detected (data not shown), ruling out the possibility of spontaneous decomposition of
312 oxalate. Although Mn²⁺ is described to be cofactor for oxalate decarboxylase, the production of
313 formate was significantly reduced (around 50%) when 1 mM of the ion was present in the
314 enzymatic reaction.

315

316 ***Formate has an effect on the development of C. capitata larvae.***

317 After demonstrating that oxalate decarboxylase has enzymatic activity, a new set of bioassays
318 was performed in order to test whether the ingestion of formate has any effect on *C. capitata*
319 larvae. For this experiment, 100 mM ammonium formate was included in the larval artificial
320 diet. As a control, 100 mM sodium oxalate was also included in the bioassay. In parallel,
321 solubilised OxdD (5 mg/well) with and without oxalate and a whole culture of *B. pumilus*
322 strain 15.1, with and without oxalate were also assayed in order to determine if the
323 combination of these elements showed any effect on toxicity (Table 3). The presence of
324 oxalate or formate in the diet showed twice the mortality of the water control. However, while
325 no effect on larval size was observed in oxalate bioassays compared to the control, a
326 substantial reduction was noticed when formate was present (larvae did not progress further
327 than first instar), indicating that formate interfered in larval development. When solubilised
328 OxdD (5 mg/well) was included in the diet with or without oxalate, similar mortalities were
329 obtained (around twice that of the water control). No differences in mortality were observed
330 when *B. pumilus* 15.1 strain was assayed either in the presence/absence of oxalate (around
331 three times more mortality than control). These results seem to indicate that the addition of
332 oxalate to the larval diet has no mayor effects on *C. capitata* mortality, either when it was
333 bioassayed alone or together with solubilised crystals/whole *B. pumilus* culture. However,
334 when the formate was present in the diet, larvae were highly undeveloped.

335

336 Discussion

337 In this work we have characterised the parasporal crystals of *B. pumilus* strain 15.1 and
338 shown them to consist of a member of the oxalate decarboxylase family of proteins. To our
339 knowledge, this is the first example of a member of an enzyme family found in parasporal
340 crystals.

341 In order to establish the location of the gene encoding the parasporal crystals of *B. pumilus*
342 15.1, both plasmids of the strain (Garcia-Ramon et al., 2015b) were removed. The
343 conventional plasmid curing methods, involving culture at high temperature and/or in the
344 presence of replication-interfering chemical compounds, have been applied to many bacteria
345 (Hara et al., 1982; Ward and Ellar, 1983; Mahillon et al., 1988; Sivropoulou et al., 2000).
346 Unfortunately, these techniques are not successful in all strains (Rajini Rani and Mahadevan,
347 1992; Feng et al., 2013). In fact, using the most conventional treatments (Ward and Ellar,
348 1983; Mahillon et al., 1988; Ghosh et al., 2000; Molnar et al., 2003) we were not able to isolate
349 a plasmid-free variant of *B. pumilus* 15.1. We assayed sub-inhibitory concentrations of SDS,
350 acridine orange and promethazine combined with high temperature (42°C), but plasmids
351 were not eliminated (data not shown). Based on previous studies, it was proposed that the
352 cell wall/cell membrane could serve as a barrier resulting in inefficient plasmid elimination
353 (Spengler et al., 2003). Hence, the curing strategy developed here was based on obtaining
354 spheroplasts of the cells before the treatment with the replication-interfering compounds.
355 The strategy was highly efficient compared to the conventional methods used for spore-
356 forming bacteria and was faster, as no successive culturing steps were needed. The method
357 described here could represent a useful approach in those strains resilient to plasmid loss
358 using conventional methods, especially in Gram-positive bacteria (we note that *B. pumilus*
359 may be tolerant to higher levels of acridine orange than other species and that this sensitivity
360 should be determined before carrying out this step at an appropriate permissive
361 concentration). Our experiments demonstrated that the *oxdD* gene of *B. pumilus* strain 15.1

362 was located on the chromosome. Although many genes encoding crystals (such as Cry toxins)
363 are encoded by plasmids, there are some encoded in the chromosome (Hu et al., 2008; Wang
364 et al., 2014). The cured *B. pumilus* strain 15.1C, showed a parasporal crystal production
365 approximately double that of the wild type strain. This may indicate that either the small
366 plasmid pBp15.1S or the megaplasmid exerts some kind of direct or indirect regulation on the
367 expression of the *oxdD* gene. Most of the CDSs on these plasmids represent hypothetical
368 proteins but the strain 15.1 genome contig 48, here shown to be part of the megaplasmid in
369 this strain, does appear to encode a YdeB-like putative transcription factor, an HTH-type MerR
370 family transcriptional regulator, a potential RNA binding regulator of transcription that is Hfq-
371 like, and a response regulator protein; although no link between these CDSs and OxDD
372 production has yet been established. The megaplasmid also appears to encode the 17 kDa
373 YuaB protein, which has homologs in *B. subtilis* and a hypothetical protein, BPUM_1610 in
374 *B. pumilus* SAFR-032. In *B. subtilis*, YuaB is a small, secreted protein that is localized at the cell
375 wall, plays a role during biofilm formation (Ostrowski et al., 2011) and is responsible for
376 forming a layer on the surface of the biofilm making it hydrophobic (Kobayashi and Iwano,
377 2012). In contrast to *B. pumilus* 15.1, in *B. subtilis* the *yuaB* gene appears to be encoded
378 chromosomally.

379

380 Oxalate decarboxylase, is a member of the cupin family of proteins, which has enzymatic
381 members but also includes non-enzymatic proteins including seed storage proteins. The
382 *B. pumilus* 15.1 oxalate decarboxylase, along with storage proteins such as canavalin and
383 phaseolin is a bicupin as it has 2 beta sandwich cupin domains (Tanner et al., 2001)(Figure 2)
384 each one containing one manganese binding site (Anand et al., 2002). The seed proteins are
385 known to show proteinase resistance, as seen for the protein described here. The protein
386 from *B. pumilus* crystals appears to form a hexameric complex, consistent with the oxalate

387 decarboxylase from *B. subtilis* that in solution (Svedružić et al., 2007) and in X-ray
388 crystallographic analysis (Anand et al., 2002) also forms hexamers.

389 Oxalate decarboxylase (EC 4.1.1.2) catalyzes the conversion of oxalate to formate and carbon
390 dioxide. The first bacterial oxalate decarboxylase was identified in *B. subtilis* (OxdC, formerly
391 known as YvrK) as a cytosolic enzyme (Tanner and Bornemann, 2000). Subsequently, a
392 second hypothetical protein (YoaN) from *B. subtilis* exhibited oxalate decarboxylase activity
393 and was named OxdD (Tanner et al., 2001), which was found to be present in the interior
394 layer of the spore coat (Costa et al., 2004). In *B. subtilis*, OxdC and OxdD are spore-associated
395 proteins (Kuwana et al., 2002) and the recombinant proteins overexpressed in *E. coli* are
396 soluble showing oxalate decarboxylase activity only when expressed in the presence of
397 manganese salts (Tanner et al., 2001). We have demonstrated that the accumulation of the
398 *B. pumilus* 15.1 oxalate decarboxylase is dependent on the Mn^{2+} concentration in the medium,
399 consistent with putative promoter elements identified upstream of the gene.

400 The oxalate decarboxylase crystals were found to solubilize at low temperature (-20°C), a
401 phenomenon that has not previously been described for a crystal protein. This is interesting
402 in light of the fact that toxicity of the original *B. pumilus* 15.1 strain was dependent on the
403 incubation of the whole culture at low temperature for at least 4 days (Molina et al., 2010). In
404 addition, oxalate decarboxylase parasporal crystals purified from *B. pumilus* 15.1 were not
405 significantly toxic in diet contamination assays against *C. capitata* larvae but a slight increase
406 of toxicity (2-3 times) was observed when solubilized protein was used (Table 1 and Table 3).

407 Although the oxalate decarboxylase protein is not able to induce the mortality of *C. capitata*
408 larvae by itself, we cannot rule out the possibility that this protein may play some role in this
409 process as other virulence factors could be necessary for full toxicity. There are few reports in
410 the literature of oxalate decarboxylase in relation to virulence. The substrate for this enzyme
411 (oxalic acid or oxalate) is associated with several plant pathogenic fungi from the genus
412 *Sclerotinia* (Bateman and Beer, 1965; Kritzman et al., 1977; Magro et al., 1984). Although the

413 exact mechanism of oxalic acid as a virulence factor is not completely understood, its ability to
414 chelate calcium ions, or to change pH, favoring some cellulolytic enzymes (Lumsden, 1979) or
415 to act as a plant defense inhibitor (Mayer and Harel, 1979; Ferrar and Walker, 1993) seems to
416 help the fungi to invade host plants. Pseudomonad-like bacterial strains synthesising oxalate
417 degrading enzymes (Dickman and Mitra, 1992) are reported to prevent *Sclerotinia*
418 *sclerotiorum* infections in plants by removing the fungal virulence factor oxalate. Oxalate
419 decarboxylase has been used in biological control of fungal plant diseases (Kesarwani et al.,
420 2000; Dias et al., 2006) making transgenic plants resistant to fungal pathogens.

421 The fact that the oxalate decarboxylase is overexpressed in *B. pumilus* 15.1 suggests an
422 important role for the bacterium. We have demonstrated that oxalate decarboxylase present
423 in *B. pumilus* 15.1 crystals shows enzymatic activity when solubilised, as formate production
424 was detected in *in vitro* enzymatic assays. The action of oxalate decarboxylase on its only
425 described substrate, oxalate (Brenda database (Schomburg, 2015)), could produce a
426 significant amount of formate when *B. pumilus* 15.1 is bioassayed and this could explain the
427 toxicity of the strain toward *C. capitata* larvae. Formate is well known for being a compound
428 toxic for insects and other arthropods and higher organisms (Elzen et al., 2004; Chaskopoulou
429 et al., 2009; Underwood and Currie, 2009; Chen et al., 2012; Chen et al., 2013) and we have
430 shown it to have a particularly detrimental effect on *C. capitata* larvae development. The
431 origin of the oxalate substrate for the enzyme to produce formate in the environment is not
432 known. The production of oxalate in bacteria is not a very frequent characteristic but in a few
433 cases its production has been related with virulence. This has been demonstrated for
434 *Burkholderia glumae*, a plant pathogen that causes seedling and grain rot via the production of
435 oxalate (Li et al., 1999). Although we cannot state definitively whether strain 15.1 is able to
436 produce oxalate, the genome data for this strain (Garcia-Ramon et al., 2015a) does not appear
437 to exhibit genes encoding ascorbate 2,3 dioxygenase (which can produce oxalate from L-
438 ascorbate), (S)-hydroxyl acid dehydrogenase (which can produce oxalate from glyoxalate) or

439 oxalate CoA transferase and glyoxylate dehydrogenase (which together can produce oxalate
440 from glyoxylate via oxalylCoA). The genome does, however, encode a putative
441 oxalate:formate symporter in the MSF family, which is present in other *B. pumilus* genomes
442 and is conserved in other bacilli but is found in few species outside this genus, so we could
443 speculate that the strain could utilise oxalate from the medium and use oxalate decarboxylase
444 to produce formate as a virulence factor. However, our data showed that an external supply of
445 oxalate in the larval diet seems not to have any effect on toxicity. Clearly many questions still
446 remain unanswered in the mode of action of *B. pumilus* strain 15.1 but this work represents a
447 step forward in the understanding of this bacterium in relation to putative novel virulence
448 factors that may be used by entomopathogenic bacteria. Characterization of the kinetics of the
449 enzyme and further investigations of its relationship with toxicity will be undertaken in
450 further studies.

451

452

453 **Experimental Procedures**

454 ***Bacterial strain and growth conditions***

455 The bacterial strain used in this study was *Bacillus pumilus* 15.1 (Molina et al., 2010). Luria-
456 Bertani (LB) medium was routinely used for growing bacteria. When sporulation was
457 required, T3 medium (Travers et al., 1987) was used and incubation was at 30°C for 72 h at
458 240 rpm. Modified T3 medium was also used with different concentrations of MnCl₂ (ranging
459 from 0 to 0.5 g/L).

460

461 ***Protein expression profile determination under different conditions***

462 *B. pumilus* 15.1 was grown in 3 mL of LB at 30°C and 240 rpm overnight and used to inoculate
463 50 mL of T3 medium for growth under the conditions described above for 72 h. Samples
464 (1 mL) were centrifuged for 1 min at 16,000 x g. Pellets were resuspended in 50 µL of PBS,

465 analyzed by SDS-PAGE and stained with Coomassie brilliant blue, according to standard
466 procedures. Precision Plus Protein™ Standards (Bio-rad) molecular weight marker was used
467 in all SDS-PAGE gels.

468

469 ***Discontinuous sucrose gradient***

470 To isolate the parasporal crystals, sporulated cultures (72 h of incubation) grown in T3
471 medium were subjected to the procedure described by Garcia-Ramon *et al.* (2016) for
472 discontinuous sucrose gradient separation.

473

474 ***Protein analysis by 2D gel electrophoresis***

475 Analyses by 2-dimensional (2D) gel electrophoresis were carried out according to the
476 manufacturer's recommendations (Biorad). Briefly, 15 µL of each protein sample were mixed
477 with 115 µL of re-hydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 10 mM DTT and
478 0.2% ampholytes) and loaded onto IPG strips (Ready Strip™ IPG Strips 11 cm, pH 3-10, Bio-
479 Rad). The strips were re-hydrated at 20°C for 16 h (passive rehydration) in a Protean® IEF
480 Cell (Bio-Rad). Isoelectric focusing (IEF) was carried out using the following four-step
481 program: (i) 250 V for 1 h in a linear mode; (ii) 4,000 V for 2 h in a linear mode; (iii) 4,000 V
482 until 18,000 Vh in a rapid mode; 500 V until 50 µA per strip in a rapid mode. After IEF, strips
483 were equilibrated for 10 min in equilibration buffer I (6 M urea, 0.375 M Tris-HCl pH 8.8, 2%
484 SDS (wt/vol), 20% glycerol (vol/vol)) containing 130 mM DTT, followed by an incubation in
485 equilibration buffer II, containing 135 mM iodoacetamide instead of DTT, for 10 min. Proteins
486 were then separated by their molecular weight by placing the strip on the top of a 12% SDS-
487 PAGE in a vertical electrophoretic unit (Bio-Rad). Electrophoresis was performed at 120 V for
488 60 min. Two dimensional gels were stained with Coomassie blue.

489

490 ***Solubilization of crystals and protease treatment***

491 Fractions from a discontinuous sucrose gradient containing most of the crystals produced by
492 *B. pumilus* 15.1 were kept frozen at -20°C until use. To determine protease stability of the 45
493 kDa protein, the sample was thawed on ice and centrifuged at 13,000 rpm for 3 min and the
494 supernatant was collected in a fresh tube. Protein concentration was determined in the
495 supernatant using Bradford's reagent (Sigma), following the manufacturer's
496 recommendations and using bovine serum albumin BSA (Sigma) as a standard. Supernatant
497 fractions were incubated with four different proteolytic enzymes: trypsin, chymotrypsin,
498 papain and "Proteinase from *Bacillus subtilis*" (cat No. 96887) from Sigma. Buffers and
499 incubation temperatures for each enzyme were chosen according the instructions provided
500 by the supplier. The standard ratio used for protease treatment was 10:1 (w/w)
501 (protein:protease), although other ratios were tested. Samples were incubated for 1 h and a
502 BSA control was carried out in parallel to verify protease activity. A sample without proteases
503 was also incubated under the same conditions as a negative control. For comparative
504 purposes, the solubilized Cry1Aa13 (expressed in *Escherichia coli* from plasmid pCP10 (Pigott,
505 2006) was also digested at the same protein:trypsin ratios (between 1:1 to 1:500,
506 protein:trypsin). All the digested proteins were analyzed by SDS-PAGE.

507

508 ***Transmission electron microscopy***

509 Fresh aliquots from the sucrose gradient fractions were pelleted and washed following the
510 methodology previously described (Garcia-Ramon et al., 2016) and sent to the "Biological
511 Sample Preparation Laboratory" at the Scientific Instrumentation Center of the University of
512 Granada (CIC-UGR) for processing. Samples were observed under a Transmission Electronic
513 Microscope (LIBRA 120 PLUS from Carl Zeiss SMT) in the Microscopy Service of the CIC-UGR.
514 Ten images of 12.6 µm in size were used to determine the crystal:spore ratio.

515

516 ***Plasmid curing procedures***

517 Three procedures reported in the literature were tested for the curing of the
518 extrachromosomal elements present in the strain *B. pumilus* 15.1. In the first place, the
519 methods described by Ward and Ellar (1983) and Mahillon et al. (1988), based on culturing
520 the strain at high temperature were used with slight modifications. *B. pumilus* strain 15.1 was
521 grown in 3 mL LB for 24 h at 42°C and 240 rpm. Successive dilutions of the culture (1:100)
522 into fresh medium were made after 12 h of incubation during a total period of 72 h. The
523 second method tested was performed as described above, with the difference that LB medium
524 was supplemented with 0.002% SDS (Sivropoulou et al., 2000). In the third procedure, the
525 *B. pumilus* 15.1 strain was grown in LB supplemented with 0.03% acridine orange or 0.12%
526 promethazine for 24 h, either at 30°C or at 42°C. Bacterial cultures were transferred (1:100
527 dilution) into fresh LB medium supplemented with the interfering compounds every 12 h for
528 5 days.

529 Cells derived from these procedures were plated on LB medium and incubated for 12-24 h at
530 30°C. Randomly selected colonies were used for total DNA extraction using the methodology
531 described by Reyes-Ramirez and Ibarra (2008). Total DNA was analyzed by electrophoresis
532 in a 0.8% (wt/vol) agarose gel with SYBR Green from Invitrogen.

533 In addition to the standard methods, above, we also developed a novel curing strategy. For
534 this, *B. pumilus* 15.1 was cultured in 5 mL of LB medium to an optical density at 600 nm of 0.9
535 to 1.1. One millilitre of the culture was pelleted at 16,000 x g for 1 min. The pellet was
536 resuspended in 1 mL PBS containing 2% (wt/vol) lysozyme and 20% (wt/vol) sucrose, and
537 was incubated at 37°C for 90 min. In this period of time, more than 90% spheroplast
538 formation was achieved as monitored under the microscope. The spheroplast suspension was
539 diluted 1:100 in LB medium supplemented with 0.03% acridine orange or 0.12%
540 promethazine and cultured at 30°C and 240 rpm for 48 h until growth was observed. Serial
541 dilutions were plated on LB plates and incubated at 30°C overnight.

542

543 ***Plasmid copy number determination***

544 Plasmid copy number was determined by quantitative real time PCR as previously described
545 (Garcia-Ramon et al., 2015b). Briefly, total DNA was used to amplify the *smc* gene that is
546 present in a single copy on the chromosome with *smc_F* and *smc_R* primers, and *orf7_F* and
547 *orf7_R* primers were used to amplify a unique region in the pBp15.1S plasmid.

548

549 ***Southern blot analysis***

550 Total DNA was electrophoresed on a 0.8% (wt/vol) agarose gel and stained with ethidium
551 bromide and transferred to a nylon membrane. The PCR product (855 ng) amplified with
552 *orf7_F* and *orf7_R* primers (Garcia-Ramon et al., 2015b) and cleaned with QIAquick® PCR
553 Purification kit (Qiagen) were used as a probe for the pBp15.1S plasmid. DNA labelling,
554 transfer and fixation to the membrane, hybridization and immunological detection were
555 performed with a DIG DNA Labeling and Detection Kit (Roche No. 11093657910) following
556 the instructions provided by the supplier.

557

558 ***Mass spectrometric analysis of protein samples***

559 Bands or spots identified for analysis from the 1D or 2D SDS-PAGE gels were individually
560 excised and sent to “Centro de Investigación Principe Felipe”, Valencia-Spain, for the peptide
561 identification by Matrix-Assisted Laser Desorption Ionization-Time Of Flight Mass
562 Spectrometry (MALDI TOF-MS). Digestion products were analyzed by MALDI MS (4700
563 Proteomics analyser of the Applied Biosystems). Searches of the *B. pumilus* 15.1 genome
564 (Garcia-Ramon et al., 2015a) and public databases were performed using MASCOT search
565 engine (Matrix-Science, London, UK). The services from “SCSIE University of Valencia
566 Proteomics Unit” and “CBMSO Protein Chemistry Facility” that belong to the ProteoRed
567 Proteomics Platform were also used. At the SCSIE University of Valencia Proteomics Unit a

568 MALDI-TOF MS/MS analysis (5800 MALDI TOFTOF ABSciex) was performed. The MS and
569 MS/MS information was analyzed by MASCOT via the Protein Pilot (ABSciex). Database
570 search was performed on NCBI nr.

571 At the CBMSO Protein Chemistry Facility (Madrid) a Liquid chromatography tandem mass
572 spectrometry (LC-MS/MS) analysis (Orbitrap-LTQ-Velos-Pro) was performed and the search
573 was made on UniProt-*Bacillus* and UniProt-*Bacillus pumilus* databases, using Proteome
574 Discoverer 1.4 software.

575

576 ***N-terminal amino acid sequencing***

577 The solubilized and trypsinized protein of 45 kDa was separated in a 12% acrylamide SDS
578 PAGE gel with Tris Tricine running buffer. Separated proteins were blotted onto PVDF
579 membrane using a semi-dry transfer blotter. N-terminal sequencing was performed by
580 Abingdon Health Laboratory Services, Birmingham, UK.

581 The sequence obtained was compared with protein sequences from the genome of *B. pumilus*
582 15.1 (GenBank LBDK000000000.1) (Garcia-Ramon et al., 2015a).

583

584 ***Size exclusion chromatography***

585 Soluble oxalate decarboxylase protein from *B. pumilus* strain 15.1 was analysed by size
586 exclusion chromatography using a HiLoad 16/600 Superdex 200 preppacked column (GE
587 Healthcare) in 50 mM sodium phosphate (pH 5.0), 300 mM NaCl using an AKTAPure 25
588 system (GE Healthcare). The molecular weight of oxalate decarboxylase in solution was
589 determined by reference to a calibration curve obtained on the same column with gel
590 filtration standards (BioRad).

591

592 **Primer design and PCR amplification of the hypothetical protein YuaB**

593 To PCR amplify the *yuaB* gene, the primers YuabF (5'
594 AAAAAGATCTAACCAAATGCGCTATTCCCC 3') and YuabR (5'
595 AAGAATTCCTTTGTCAACAATCTGAAGCGC 3') were designed based on the sequence from
596 *B. pumilus* 15.1 (Garcia-Ramon et al., 2015a). Total DNA from the wild type and the cured
597 strain were used under the following PCR conditions: 95°C for 5 min, followed by 30 cycles of
598 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min and then a final extension at 72°C for 5 min.
599 Amplification was checked by electrophoresis on a 1% (wt/vol) agarose gel.

600

601 ***C. capitata* larval bioassays**

602 Bioassays with *B. pumilus* strain 15.1 were performed as described previously (Molina et al
603 2010). When ammonium formate or sodium oxalate were bioassayed, solid powder from
604 these compounds was dissolved in the diet to a final concentration of 100 mM. The
605 insecticidal activity of insoluble parasporal inclusion suspensions obtained from *B. pumilus*
606 15.1 was tested at a cell density approximately 40 times greater than the original culture
607 following Molina *et al.* (2010) with some modifications. When solubilised, oxalate
608 decarboxylase was assayed at 10 µg/mL of diet (5 µg/well). Briefly, 100 µL of the samples
609 were dispensed into each well and mixed with 500 µL of artificial diet. One larva of *C. capitata*
610 was placed in each well. The bioassays were performed in 48-well sterile Cellstar microplates
611 (Greiner Bio-one) at 25°C. Deionized water was used as negative control. All bioassays were
612 performed at least twice using different cultures or crystal samples obtained from separate
613 cultures and gradients. In all bioassays mortality was recorded 10 days after the beginning of
614 the bioassay.

615 **Enzymatic assays**

616 The activity of oxalate decarboxylase was evaluated by the production of formate using two
617 methods. In the first, the oxalate decarboxylase activity assay kit (Sigma Aldrich) was used

618 according to the manufacturer's instructions. Results were compared to a range of
619 concentrations of formate and with the activity of an oxalate carboxylase positive control
620 (both provided in the kit). The second assay detected formate production by nmr. Briefly,
621 300 µl of sodium phosphate buffer (100 mM, pH 5.0) was mixed with 200 µl of sodium oxalate
622 (300 mM, pH 5.0) in a final volume of 600 µl containing 0, 5 or 10 µg of oxalate decarboxylase
623 enzyme (previously purified by sucrose gradient and solubilized in Milli Q water at low
624 temperature as described above). When indicated, 1 mM MnCl₂ was included in the assay. The
625 mixture was incubated for 2 h at 37°C and the reaction was stopped with 1 mL of sodium
626 phosphate buffer (150 mM, pH 9.5). Then, methanol (reagent grade, Sharlau) was added to
627 each sample to a final concentration of 5 mM as an internal reference for ¹H-NMR analysis.
628 Samples were analysed in a Varian Direct Drive Spectrometer of 500 MHz at the Centro de
629 Instrumentación Científica of the University of Granada. Spectra were obtained under fully
630 relaxed conditions and the water signal was suppressed. The area of each peak was integrated
631 using MestReNova 9.0 software taking the methanol signal as an internal reference.

632

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Table 1: Mortality results obtained after 10 days in *C. capitata* larvae bioassays using insoluble and soluble crystals obtained from *Bp* 15.1 after sucrose gradient purification and incubation at -20°C for solubilisation. The increase in toxicity compared to the negative control was also calculated.

Bioassay	% Mortality	Fold increase
H ₂ O (-ve control)	6.25 ± 2	1
Untreated crystals from <i>Bp</i> 15.1	4.2 ± 1	0.6
Solubilized crystals from <i>Bp</i> 15.1	18.8 ± 3	3.0
Pellet remaining after solubilisation	6.79 ± 2	1.1

Table 2: Integral value of peaks at 8.40 ppm (corresponding to formate) and estimated formate concentration using methanol as internal reference. Formate production was evaluated in the presence (1 mM) and the absence of Mn²⁺ ions and with different amounts of oxalate decarboxylase enzyme.

µg of enzyme	- Mn ²⁺		+ Mn ²⁺	
	Integral value ^a	Formate ^b concentration (mM)	Integral value ^a	Formate ^b concentration (mM)
0	0	0	0	0
5	0.19 ± 0.00	0.31 ± 0.00	0.11 ± 0.00	0.18 ± 0.01
10	0.42 ± 0.01	0.60 ± 0.02	0.18 ± 0.00	0.29 ± 0.00

^a Mean of the integral values obtained in two different enzymatic assays.

^b Estimated formate concentration using 5 mM methanol as internal reference.

Table 3: Mortality results obtained after 10 days in *C. capitata* larvae bioassays using different chemicals (oxalate and formate), *B. pumilus* 15.1, and soluble oxalate decarboxylase. The increase in toxicity compared to the negative control was also calculated.

Bioassay	% Mortality	Fold increase
H ₂ O (-ve control)	14.35	1
Formate (100 mM)	27.35 ± 3 ^a	1.8
Oxalate (100 mM)	29.84 ± 2	2.0
Oxalate (100 mM) + Soluble OxdD ^b	28.3 ± 24	1.9
Soluble OxdD ^b	27.19 ± 6	1.8
<i>Bp</i> 15.1	41.67 ± 10	2.8
<i>Bp</i> 15.1+oxalate (100 mM)	44.79 ± 25	3.0

^a The body size of larvae found in this bioassay was similar to first instar larvae.

^b The amount of soluble oxalate decarboxylase (OxdD) was 5 µg/well (10 µg/mL of diet)

830 **Figure 1. Two-dimensional electrophoresis of a fraction obtained from the sucrose gradient of a**
831 ***B. pumilus* 15.1 culture.** The pH (pI) range is shown horizontally and molecular weight (kDa) is shown
832 vertically. The pI ranged from 3 to 10. Arrow A shows pI 5.5; Arrow B shows pI \geq 10.

833

834 **Figure 2. OxdD model.** The model of *B. pumilus* 15.1 OxdD was produced using Swiss model. The two
835 conserved Mn²⁺ binding sites (H96, H98, E102 and H274, H276, E281) are coloured red and shown with sticks
836 and dots. The symmetry of the molecule with its two cupin domains (left and right) can be seen clearly.

837

838 **Figure 3. Protein profile of the pellet fractions of *B. pumilus* 15.1 cultures grown on T3 medium in the**
839 **presence of different concentrations of MnCl₂.** The standard conditions for MnCl₂ were 5 mg/L (lane 2). Lane 0
840 shows a pellet fraction of a culture without MnCl₂, lane 1 with 0.5 mg/L MnCl₂, lane 3 with 50 mg/L MnCl₂, and lane 4
841 with 0.5 g/L MnCl₂. Lane M shows a molecular weight marker (Precision Plus Bio-rad) in kDa. The arrow shows the
842 oxalate decarboxylase protein.

843

844 **Figure 4. SDS-PAGE analysis of the pellet and supernatant fraction of oxalate decarboxylase crystals**
845 **obtained from a fresh sucrose gradient and kept at room temperature RT (panel A) or low temperature**
846 **(Panel B).** The incubation at -20°C solubilized the 45 kDa oxalate decarboxylase over time while during
847 incubation at RT the protein remained in the insoluble fraction. Lanes S represent the supernatant fractions and
848 lanes P represent the pellet fractions of the samples. The arrows indicate the oxalate decarboxylase protein.
849 Lanes M show the Precision Plus Bio-rad molecular weight marker in kDa.

850

851 **Figure 5. SDS-PAGE analysis of the oxalate decarboxylase and the Cry1Aa13 digested with different**
852 **proteases.**

853 Panel A shows the oxalate decarboxylase digested with trypsin (lane 2), chymotrypsin (lane 3), papain (lane 4)
854 and “proteinase from *B. subtilis*” (lane 5). Panel B and C shows digestions of the oxalate decarboxylase with
855 trypsin (Panel B) and chymotrypsin (Panel C) at protein:protease ratios 1:1 (lanes 2), 1:10 (lanes 3), 1:50 (lanes
856 4), 1:100 (lanes 5) and 1:500 (lanes 6). Panel D shows the digestion of Cry1Aa13 at the same protein:protease
857 ratios as Panel B. As control, lanes 1 show the soluble proteins with no protease treatment. Lanes M show the
858 molecular mass marker (Precision Plus Bio-rad) in kDa.

859

860 **Figure 6. DNA electrophoresis in 0.8% agarose gel of total DNA extracted from several *B. pumilus* 15.1**
861 **variants.** Wild-type strain is shown in lanes 1 and 2. Variants obtained with the prior formation of spheroplasts
862 are shown in lanes 3 (treated with acridine orange) and 4 (treated with promethazine). Lanes 5 and 6 show two
863 variants treated with acridine orange and promethazine respectively without lysozyme treatment. M: Molecular
864 weight marker (HyperLadder I from Bioline) in base pairs. White arrows indicate the megaplasmid (pBp15.1B)
865 and the plasmid (pBp15.1S) respectively, and black arrow indicates the chromosomal DNA.

866
867 **Figure 7. DNA electrophoresis (Panel A) and Southern blot (Panel B) of total DNA from *B. pumilus* 15.1**
868 **wild type (lanes 1) and *B. pumilus* 15.1C (lanes 2).** Electrophoresis was performed in a 1% agarose gel and
869 stained with ethidium bromide. Southern blot was performed with a DIG labeled probe designed in the *orf7* of the
870 plasmid pBp15.1S (Garcia-Ramon et al., 2015b). M: Molecular weight marker (HyperLadder I from Bioline) in
871 base pairs. The white arrows indicate the megaplasmid, the chromosome and the plasmid from top to bottom,
872 respectively.

873
874 **Figure 8. SDS-PAGE analysis of the pellets from *B. pumilus* 15.1 and *B. pumilus* 15.1C cultures.**
875 White arrows show the oxalate decarboxylase protein at 45 kDa in the wild type (lane 1) which is more intense
876 in the cured strain (lane 2) and the 17 kDa protein present only in the wild type strain. Lane M shows the
877 molecular weight marker (Precision Plus Bio-rad) in kDa.

878
879 **Supplementary Figures**

880 **Figure 1S: Multimeric form of *B. pumilus* 15.1 Oxalate decarboxylase determined by size-exclusion**
881 **chromatography.** The column was calibrated with the gel filtration standards from Bio-Rad (grey circles)
882 vitamin B12 (1.3 kDa) (1), myoglobin (17 kDa) (2), ovalbumin (44 kDa) (3), γ -globulin (158 kDa) (4) and
883 thyroglobulin (670 kDa) (5). Red circles represent the theoretical elution volume for the OxdD monomer (a),
884 hexamer (b) and heptamer (c). The blue circle represents the experimental elution volume obtained for *B.*
885 *pumilus* 15.1 OxdD.

886
887 **Figure 2S: Representative spectra obtained in the H-NMR analysis.** The spectra were obtained from oxalate
888 decarboxylase enzymatic reactions using 5 μ g (panels A and C) and 10 μ g of enzyme (panels B and D). Reactions
889 were performed in the absence (panels A and B) and in the presence (panels C and D) of 1 mM of Mn^{2+} .
890 Integration values for formate (8.404 ppm) were calculated using methanol (3.3 ppm) as an internal reference.